Stromal Estrogen Receptor-α Promotes Tumor Growth by Normalizing an Increased Angiogenesis

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Abstract

Estrogens directly promote the growth of breast cancers that express the estrogen receptor α (ERα). However, the contribution of stromal expression of ERα in the tumor microenvironment to the protumoral effects of estrogen has never been explored. In this study, we evaluated the molecular and cellular mechanisms by which 17β-estradiol (E2) impacts the microenvironment and modulates tumor development of ERα-negative tumors.

Introduction

Estrogen receptor α (ERα) holds a key position for diagnosis and treatment of breast cancers. Indeed, its expression by breast cancer cells dictates the use of endocrine therapies, such as tamoxifen and aromatase inhibitors, that blocks estrogen activity (1). It is consistent with the fact that estrogen could directly promote the growth of these tumors classified as ERα positive. However, evidence of an increasing number of data suggests that estrogen could promote the growth of ERα-negative tumors.

Discussion

Interestingly, various clinical and experimental data support this putative implication. Indeed, breast tumors are classified as ERα-positive even when only 1% of breast cancer cells express ERα, questioning the mechanisms accounting for the efficacy of targeting a so rare cell population. In addition, ovariectomy seems to be efficient to decrease long-term recurrence risk and mortality of breast cancer classified as ERα-negative and as ERα-positive (9, 10). These data point out that, beside cancer cell-associated ERα, additional E-dependent mechanisms interfere with the efficacy of endocrine therapy. This is supported by the fact that tamoxifen response rates were low but still can be found in ERα-negative/progesterone receptor-negative tumors (<10%; ref. 12). However, these ERα-negative breast tumors and particularly triple-negative breast cancer are no longer treated with endocrine therapy and exhibit poor prognosis (13). In immunodeficient animal models, pregnancy or 17β-estradiol (E2) supplementation was found to accelerate the growth of human ER-negative breast cancer cells (14, 15). E2 has been shown to impact angiogenesis (4, 14); however, there is still a paucity of information with regard to the specific molecular mechanisms by which E2 could impact tumor microenvironment and angiogenesis. Particularly, the
contribution of host ERα and ERβ remains poorly documented (16, 17). To improve the management of cancer patients and particularly of women, it is mandatory to get mechanistic information with regard to the various ways by which E2 could impact tumor development and progression. This is of particular interest knowing that estrogen exert multiple functions in cells, depending on targeted cell type and organ (6, 18).

The aim of this work is to characterize which ER expressed by the tumor microenvironment and which ER-expressing cells are important for tumor development. Because the innate and adaptive immunity make crucial contributions to tumor development and to the antitumor effects of conventional radiation and chemotherapy, we used immunocompetent animals (19, 20). In models of ERα-negative tumor cell lines injected to syngeneic immunocompetent mice, we report here that E2 induces tumor growth through an increase and improvement of angiogenesis by triggering stromal cells via Tie2-dependent ERα.

Materials and Methods

Cell culture

Mouse B16K1 (MHC class I–positive B16F10) melanoma cell line was used as previously described (21). Mouse Lewis lung carcinoma cell line (LL2, #CRL-2531), mouse breast tumor cells 4T1 (#CRL-2539), and mouse endothelial cell line derived by SV40 (#CRL-2181) were purchased and authenticated from American Type Culture Collection, routinely cultured as recommended by manufacturer and used from passages 3 to 8. B16K1, LL2, and 4T1 were last authenticated in February 2012 by Leibniz-Institut DSMZ GmbH.

Animals

Female C57BL/6J and Balb/C mice (4 weeks old) were obtained from Charles River Laboratories. ERα−/−, ERβ−/− mice, Tie2-Cre+/−/ERα-flox (Cre+/−) mice, and their control wild-type (WT) littermates ERα−/−, ERβ+/−, and Tie2-Cre+/−/ERα-flox (lox+/−) mice were generated as described previously on C57BL/6J background (22, 23).

In vivo tumor models

Four-week-old female mice were ovariectomized (OVX) to prevent endogenous estrogen production. Two weeks before cancer cell injection, mice were implanted subcutaneously with a pellet releasing E2 (Innovative Research of America) or were sham operated (untreated control group). B16K1, LL2, or 4T1 (4 × 10⁶ cells suspended in PBS) were injected subcutaneously to both flanks of WT or transgenic C57BL/6J mice (B16K1, LL2) or of Balb/C (4T1) mice.

Harvesting of tumors

Before sacrifice, mice were anesthetized and perfused by intravenous injection of fluorescein isothiocyanate (FITC)–conjugated lectin (Vector) that was allowed to circulate for 5 minutes. The tumor vasculature was then fixed by intracardiac perfusion of 4% PFA and then embedded in paraffin or OCT.

Histologic analysis and quantification of angiogenesis, vessel perfusion, and mural cell coverage

Morphologic analyses were carried out on paraffin-embedded tumor sections, stained with hematoxylin and eosin (H&E) or immunolabeled with CD34 (AbD-serotec) or Ki67 (Dako). For fluorescent microscopy, frozen tumor sections were immunolabeled with Cy3-conjugated anti-αSMα (Sigma-Aldrich) and anti-CD31 (BD-Pharmingen), evidenced with a rat-IgG–specific biotinylated antibody (Vector Laboratories) and Cy3-conjugated Streptavidin (Sigma-Aldrich).

Confocal microscopy analysis and quantification of tumor vascular network

Frozen tumor previously perfused with FITC-conjugated lectin were cut, then counterstained and mounted with 4', 6-diamidino-2-phenylindole (DAPI)–mounting medium (Vector Laboratories). The 3-dimensional (3D) images were obtained and quantified as explained in Supplementary Fig. S1. Image analysis was carried out using the Matlab 7.9 software.

Hypoxia and necrosis assessment

Tumor hypoxia was evaluated with the Hypoxyprobe-1 kit (#HP2-100; Chemicon) following manufacturer’s guidelines.

Antibody array and qRT-PCR

Proteins from size match B16K1 tumors were extracted and analyzed on RayBio Mouse Cytokine Antibody-Array according to manufacturer’s instructions (RayBiotech). mRNA of endothelial cells were amplified on Biomark (Fluidigm). Data were analyzed with both Fluidigm Real-Time PCR analysis and ValidPrime (24) softwares.

Statistical analysis

All quantitation experiment data are expressed as mean ± SD or mean ± SEM. Statistical analysis were conducted with GraphPad Prism software. The value of P ≤ 0.05 was considered as statistically significant.

Other methods

Expanded methods are provided in Supplementary Methods.

Results

E2 promotes ER-negative cancer cell growth in ER-positive immunocompetent microenvironment

To determine whether E2 affects the growth of ER-negative cancer cells in immunocompetent mice, we injected ER-negative tumor cell lines into syngeneic C57BL/6J (B16K1, LL2) or Balb/C (4T1) mice. Both B16K1 and LL2 cells were selected because they did not express ERα (B16K1), or very poorly (LL2), as shown by immunoblotting (Supplementary Fig. S2A). Furthermore, E2 had no effect on proliferation of B16K1, nor LL2 cells, in vitro (Supplementary Fig. S2B and S2C). The 4T1 mouse breast tumor ERαIs did not express ERβ as shown by immunocytochemistry (Supplementary Fig. S2E). Nevertheless, all B16K1 (Fig. 1A) LL2 (Fig. 1B) and 4T1 (Fig. 1C) growth were increased in vivo in OVX mice treated with E2. B16K1 cells were
E2 increases angiogenesis in ER-negative tumors

E2 has been shown to increase physiologic angiogenesis (4). As angiogenesis is a key process that sustains tumor growth in the early steps of tumor development and can also be influenced by tumor size (25), the effect of E2 on tumor angiogenesis was explored at different time points to collect tumors that matched the size and E2 impregnation. Altogether, the E2-induced growth of ER-negative cancer cells is associated with increased density of intratumoral perfused vessels.

E2 improves tumor angiogenesis qualitatively

Vessel morphology was evaluated with endothelial cell marker CD34 on size match tumors. Tumor vessels from untreated mice followed serpentine course and looked tortuous with irregular and heterogeneous structures (Fig. 2A, left panels). In contrast, vessels in E2-treated mice progressed from tumor periphery through aligned orientation (Fig. 2A, right panels). Nuclei of adjacent endothelial cells were more distant from each other in E2-treated tumors as compared with the untreated ones and endothelial cells presented an elongated morphology feature of lined endothelial cells monolayer (Fig. 2A, bottom panels, pointed by arrows).

Furthermore, confocal microscopy analysis of lectin–FITC staining of thick (100 μm) tumor sections followed by 2D projection of z-slice images emphasized a major impact of E2 on tumor vessel architecture (Fig. 2B). In untreated group, vessels were uneven and dilated, whereas E2 treatment promoted a dense network of thin vessels evenly distributed with multiple branching points. Standardized computer-assisted image analysis of 3D image constructions from z-slice images of thick tumor sections allowed vessel diameter quantification and 3D architecture evaluation (Fig. 2C). This original method detailed in Supplementary Fig. S1 revealed a heterogeneous distribution of vessel diameters in untreated tumors, ranging from 0.1 to more than 20 μm. The mode, the value that occurs most frequently, of vessel diameter distribution was 11.02 ± 0.65 μm for tumors of 75 mm³ (Fig. 2D) and 12.47 ± 0.65 μm for tumors of 200 mm³.
200 mm$^3$ (Fig. 2E), respectively. In contrast, E2-treated tumors were characterized by a narrow distribution of vessel diameters remaining below 15 μm. In those E2-treated mice, a highly regular vessel network was observed, in both 75 and 200 mm$^3$ tumors, with a lower mode value [4.57 ± 0.20 (N = 4) and 4.55 ± 0.23 μm (N = 6), respectively]. Altogether, E2 improves the overall structure of tumor vascular network and regulates its organization.

**E2 improves vessel stabilization and oxygenation of ER-negative tumors**

Coverage of vessels by mural cells is a criterion of vessel stabilization and maturity (26). Double staining for lectin–FITC and the mural cell marker α-smooth muscle actin (αSMA) was quantified by computer-assisted image analysis. Little perivascular labeling was observed at the onset of tumor growth irrespective of the treatment (size match: 75 mm$^3$, Fig. 3A top panels). After 4 days (size match: 200 mm$^3$), E2 increased the

![Figure 2.](image)

**Figure 2.** E2 modifies the structure of tumor vasculature in B16K1 tumors. B16K1 tumors untreated (OVX) or treated with E2, harvested after 7, 11, or 15 days (D7-D15) for time and size match analysis. A, staining for CD34 endothelial cell marker. Scale bars, 50 μm. The 2D projections (B) and 3D images (C) from computer-assisted construction of z-sections recorded by confocal microscopy of lectin–FITC–positive vascular network; scale bars, 50 μm. D and E, vessel diameter distributions of lectin–FITC–positive vascular network; N = 3 to 6. Statistical analysis: ***, P < 0.01 E2 versus OVX.

![Figure 3.](image)

**Figure 3.** E2 improves vessel maturation and oxygenation of B16K1 tumors. B16K1 tumors untreated (OVX) or treated with E2 harvested after 7, 11, or 15 days (D7-D15) for time and size match analysis. A, double staining for lectin–FITC (green) and αSMA (red); scale bars, 67 μm. B, quantification of αSMA-positive vessel. C, variation of vessel coverage by mural cells between tumors of 75 mm$^3$ and 200 mm$^3$. For B and C, N = 5 to 8 tumors; optical fields, 8 to 12 per tumor. D, double staining for PIMO (green) and DAPI (blue); scale bars, 1 mm. E, quantification of hypoxic (PIMO positive) tumor area; scale bars, 500 μm. G, scored quantification of tumor necrosis area of size match (200 mm$^3$) B16K1 and of size match (400 mm$^3$) 4T1 tumors. B16K1: OVX, N = 16; +E2, N = 12; 4T1: OVX, N = 7; +E2, N = 8. All results are mean ± SEM. For all statistical analysis: **, P < 0.05; ***, P < 0.01; ****, P < 0.001 E2 versus OVX.
Host ERO is necessary to induce E2-dependent growth of ER-negative tumors

As ERO mediate most of the vascular effects of E2 (5), the ability of E2 to stimulate the growth of B16K1 and LL2 was assessed in ERO+/− mice and in their control ERO−/− littermates. The protumoral effect of E2 observed, to different extent, on both B16K1 and LL2 growing in ERO+/− mice was completely abrogated when the host was ERO deficient (Fig. 4A and B). In contrast, E2 still accelerated ER-negative cancer cell growth in ERO−/− deficient mice (Fig. 4C). These findings supported that host ERO is absolutely required for the E2-mediated protumoral effect on these ER-negative cancer cells, whereas EROβ is dispensable.

The effect of E2 on angiogenesis and oxygenation of ER-negative tumor requires host ERO

No significant difference of tumor vessel density was observed in ERO+/− mice in response to E2 (Fig. 4D). Quantification from 3D images of lectin−FITC−stained sections showed that the regular distribution of tumor vessel diameters elicited by E2 in ERO+/− mice was completely lost in ERO−/− mice (Fig. 4E). Clearly, in ERO+/− mice treated or not with E2, the tumor vessel network remained unchanged, irregular with large vessels displaying variable diameters up to 20 μm (Fig. 4F). Moreover, staining for the pimonidazole revealed density of αSMA-positive vessels by 2.9-fold magnitude, whereas the mural cell coverage of vessel remained unchanged in untreated animals (Fig. 3A, bottom panels, Fig. 3B and C). Confocal microscopy (Supplementary Fig. S4) and morphologic analysis of αSMA staining confirmed that the maturation of new vessels by mural cells was increased by E2 during tumor growth. In addition, under E2 treatment, vascular cell proliferation assessed by Ki-67 immunostaining (Supplementary Fig. S5) was high at the beginning of tumor growth on day 7 and decreased on day 11. By contrast, the proliferative rate of vascular cells remained unchanged in untreated group (OVX).

Figure 4. ERO is necessary to mediate the E2-dependent increase of tumor angiogenesis, oxygenation, and growth. In vivo growth curve of B16K1 (A) or LL2 (B) in ERO+/− mice and of B16K1 in ERO−/− mice (C), untreated (OVX) or treated with E2. For D–J, B16K1 cells were implanted in EROx+/− mice and collected at size match (200 mm3). D, tumor vessel density quantified by CD31-positive staining, N = 8. Vessel diameter distributions of lectin−FITC-positive vascular network in B16K1 tumors from E2-treated EROx+/− versus EROx−/− mice (E) or from untreated (OVX) versus E2-treated EROx−/− mice (F); N = 4 to 6. G, double staining for PIMO (green) and DAPI (blue); scale bars, 600 μm. H, quantification of hypoxic (PIMO positive) tumor area, N = 5 to 7. I, hematoxylin in coloration revealing necrosis area; scale bars, 500 μm. J, scored quantification of tumor necrosis area; N = 10 to 16. All results are mean ± SEM. For all statistical analysis: NS, no statistical difference (P > 0.05). *, P < 0.05; **, P < 0.01; *** P < 0.001 E2 versus OVX. BM, bone marrow.
Named Lox/Cre was abundant in ER 
E2 treatment (Fig. 4G and H). Consistently, tumor necrosis 
poietic cells (23). Because recruitment of bone marrow 
titation of ER 
E2-treated (OVX) or treated with E2, 
irradiated, then grafted with bone marrow cells from ER 
treatment, whereas those of PlGF, Notch1, and Dll4 
sVEGFR-1, thrombospondin-1 (TSP-1), and PECAM was 
sVEGFR-3, TGF 
sVEGFRs, VEGFR-3, whose expression was very low in untreat-
tants with ER 
expressed by hematopoietic 
cells to the protumoral effect of E2. Thus, ERT 
be bone marrow-derived cells ERT 
expressed by peritumoral microenvironment of 
Recent studies have shown that stromal ER 
increases and improves tumor angiogenesis, thereby decreasing 
tumor hypoxia and necrosis.

**Tie2-dependent ERα, but not bone marrow-derived cells**

**E2-driven E2-dependent ER-negative cancer cell growth**

Our results sustain that E2 acts on tumor microenvironment by modulating angiogenesis. Hence, to identify the host cells implicated in the protumoral effect of E2, we used a Tie2-specific inactivation of ERα (using Tie2-Cre+/ERTα-flox, named Cre+/; ref. 28). As expected, E2 increased B16K1 tumor growth in their control littermates Tie2-Cre-/ERTα-flox (namedlox+/). By contrast, this effect was completely abrogated in Tie2-Cre+/ERTα-flox (Cre+/) mice (Fig. 5A) showing that ERα of Tie2-expressing cells is necessary to mediate the protumoral effect of E2. In Tie2-Cre+/ mice, specific inactivation of ERα appears mostly in both endothelial and hematopoietic cells (23). Because recruitment of bone marrow-
derived cells contributes to vasculogenesis and tumor progression (29, 30), we evaluated the role of hematopoietic ERα. OVX C57BL/6j mice were lethally irradiated and successfully grafted with bone marrow cells from either ERα+/− or ERα+/+ mice, then treated or not with E2 (Supplementary Fig. S7). The B16K1 tumor growth was similarly accelerated by E2 in mice grafted with ERα+/− or ERα+/− bone marrow (Fig. 5B), suggesting that bone marrow ERα is dispensable for the protumoral effect of E2. To ascertain that bone marrow ERT 
expression (29, 30), we evaluated the role of hematopoietic ER 
expression is implicated in E2-dependent ER-negative 
cancer cell growth. A, in vivo growth curve of B16K1 injected in Tie2-
Cre+/ERTα-flox (named Cre+/x) or in Tie2-Cre-/ERTα-flox (namedlox+/x), untreated (OVX) or treated with E2, N = 10. B-D, mice were lethally 
irradiated, then grafted with bone marrow cells from ERα+/− or ERα+/+ mice and after complete bone marrow recovery, untreated (OVX) or treated with E2 before B16K1 implantation. B, in vivo growth curve of B16K1 in chimeric C57BL/6j mice, N = 16. In vivo growth curve of B16K1 in chimeric Tie2-Cre+/ERTα-flox (named Cre+/-/C−) and in Tie2-Cre-/ERTα-
flox (namedlox+/-/C−); D, N, 18. All results are mean ± SEM. For all statistical 
analysis: NS, no statistical difference (P > 0.05); *, P < 0.05; **, P < 0.01; ***; ***, P < 0.001 E2 versus OVX.

Figure 5. Tie2-dependent ERα is implicated in E2-dependent ER-negative cancer cell growth, A, in vivo growth curve of B16K1 injected in Tie2-Cre+/ERTα-flox (named Cre+/x) or in Tie2-Cre-/ERTα-flox (namedlox+/x), untreated (OVX) or treated with E2, N = 10. B-D, mice were lethally 
irradiated, then grafted with bone marrow cells from ERα+/− or ERα+/+ mice and after complete bone marrow recovery, untreated (OVX) or treated with E2 before B16K1 implantation. B, in vivo growth curve of B16K1 in chimeric C57BL/6j mice, N = 16. In vivo growth curve of B16K1 in chimeric Tie2-Cre+/ERTα-flox (named Cre+/-/C−) and in Tie2-Cre-/ERTα-
flox (namedlox+/-/C−); D, N, 18. All results are mean ± SEM. For all statistical 
analysis: NS, no statistical difference (P > 0.05); *, P < 0.05; **, P < 0.01; ***; ***, P < 0.001 E2 versus OVX.

large hypoxic areas when tumors grew in ERα−−/ mice despite 
E2 treatment (Fig. 4G and H). Consistently, tumor necrosis 
was abundant in ERα−−/ mice and not modulated by E2 (Fig. 4I 
and J). Altogether, these results showed that stromal ERα 
in vivo expression is implicated in E2-dependent ER-negative 
cancer cell growth. A, in vivo growth curve of B16K1 injected in Tie2-
Cre+/ERTα-flox (named Cre+/x) or in Tie2-Cre-/ERTα-flox (namedlox+/x), untreated (OVX) or treated with E2, N = 10. B-D, mice were lethally 
irradiated, then grafted with bone marrow cells from ERα+/− or ERα+/+ mice and after complete bone marrow recovery, untreated (OVX) or treated with E2 before B16K1 implantation. B, in vivo growth curve of B16K1 in chimeric C57BL/6j mice, N = 16. In vivo growth curve of B16K1 in chimeric Tie2-Cre+/ERTα-flox (named Cre+/-/C−) and in Tie2-Cre-/ERTα-
flox (namedlox+/-/C−); D, N, 18. All results are mean ± SEM. For all statistical 
analysis: NS, no statistical difference (P > 0.05); *, P < 0.05; **, P < 0.01; ***; ***, P < 0.001 E2 versus OVX.

E2 modulates angiogenic factor expression in ER-
negative tumors

To further characterize whether intratumoral angiogenic factors can be influenced by E2 treatment, expression of a set of proteins known to regulate angiogenesis was analyzed using an antibody array on whole-protein extracts of size matched 
tumors. Of all candidates analyzed, levels of basic fibroblast 
growth factor (bFGF) and of VEGF-D remain unchanged, whereas both VEGF-A and platelet platelet factor-4 (PF4) were 
upregulated by E2 (Fig. 6A). In addition, among the VEGF 
receptors, VEGFR-3, whose expression was very low in untreated 
tumors as compared with VEGFR-1 and VEGFR-2, was 
strongly upregulated in E2-treated tumors. As we showed that angiogenesis contributes to E2-mediated growth of ER-
negative cancer cells, we evaluated in vitro whether E2 could 
modulate the angiogenic expression profile of an endothelial ER-positive cell line, CRL-2181 (Fig. 6B). Among the set of genes analyzed, expression of VEGF-A, VEGF-D, VEGFR-1, sVEGFR-1, thrombospondin-1 (TSP-1), and PECAM was 
significantly upregulated by E2 treatment. Transcripts of VEGF-B, VEGFR-3, TGFβ1, and VCAM-1 tended to increase 
under E2 treatment, whereas those of PIGF, Notch1, and DII4 
decreased.

Altogether, these data indicated that E2 could modulate intratumoral angiogenic factor levels and that endothelial 
expression of some angiogenic factors could be impacted by 
E2 treatment.

ERα is expressed by peritumoral microenvironment of 
human breast cancers

To assess whether the expression of ERα occurs in vivo in the 
stroma of human tumors, immunohistochemistry (IHC) on 
human ERα-positive and ERα-negative breast cancer tissues 
was conducted. Close to the strong staining of malignant ERα-
positive tumor cells (Fig. 7A), a relevant ERα staining was
observed in some stromal cells. Stromal expression, detected in some fusiform-shaped fibroblast-like cells (thin arrows), was also detected in human breast tumors classified as ERα-negative tumors (Fig. 7B and C). In addition, double immunostaining for ERα combined either with CD45 (Fig. 7D) or SMA antibody (Fig. 7E) revealed that ERα-positive stromal cells were not leucocytes and were SMA negative. However, some rare costainings for ERα and CD31 (Fig. 7F) were observed in human breast cancer stroma. These data indicated that ERα immunostaining can be found in stromal cells in the microenvironment of human breast cancers irrespectively of the ERα expression by cancer cells.

Discussion

Until now, several evidences have suggested that E2 affects tumor microenvironment independently of its direct effect on tumor cell growth. However, the cellular and molecular mechanisms driving these interactions remained undetermined in immunocompetent mice. This study describes novel insights showing that E2 acts through ERα expressed by tumor microenvironment to promote tumor growth via an increased and normalized angiogenesis.

The cellular effectors of inflammation are key constituents of the microenvironment. Moreover, E2 is known to increase the production of proinflammatory cytokines, in various cell populations such as CD4+ lymphocytes (31), natural killer cells (32), and macrophages (33). First, we evidence that the protumoral impact of E2 on ERα-negative tumor cell growth was also present in Rag2−/− immunodeficient mice that lack T and B lymphocytes, indicating that these immune cells do not play a major role in the E2 effect. Second, using chimeric mice reconstituted with bone marrow from ERα−/− or ERα+/+ mice, we show that ERα of bone marrow–derived cells is not required for acceleration of B16K1 tumor growth in C57BL/6J mice by E2. This result diverges from Gupta and colleagues (14) who described that bone marrow cell recruitment was sufficient to mediate the E2-induced growth of human ER-negative tumorigenic breast epithelial cells implanted to immunodeficient mice. Major differences in the experimental protocols, that is, selective ERα ablation in bone marrow of immunocompetent animals (this work) versus coinjection of a mix of immunodeficient bone marrow cells, epithelial tumor cells, and Matrigel into immunodeficient mice (14) probably accounts for the apparent discrepancy. The present data clearly show that E2 impregnation not only increases vessel density but also improves qualitatively tumor angiogenesis.
angiogenesis, by improving vessel structure, organization, and stabilization by mural cell recruitment. This impact of E2 completely relies on host ERα, as tumor growth and vascular network in ERα−/− mice were unresponsive to E2 treatment. In tumors that grew in E2-treated host, the vessel network was dense with multiple branching points and clearly oriented. Vessels were composed of lined endothelial cells presenting flattened and elongated morphology with distant nuclei, their diameter were thin, and even resulting in a more regular shape. E2 also improved their maturation by increasing neovessel coverage by mural cells, and drastically reducing both tumor hypoxia and necrosis. Modulation of intratumoral angiogenic factors is a likely mechanism underlying this improvement of angiogenesis. Upregulation of VEGF-A by E2 was already documented (4), whereas upregulation of VEGFR-3 is of particular interest because it was recently reported to control the rapid conversion of tip cells to stalk cells during angiogenesis (34). At the endothelial cell level, transcription of various genes reported to be related to angiogenesis and vessel maturation was modulated by E2. Indeed, the increase of VEGFR-1 and sVEGFR-1 has been implicated in vessel maturation (26, 35). The antiangiogenic factor TSP-1 was also upregulated in endothelial cells by E2, a modulation already reported in T47-D and MCF-7 cells (36). Knowing the high degree of complexity by which E2 impacts cell physiology, all these data indicate that the balance between pro- and antiangiogenic factors can be modulated by E2 and contributes through paracrine interactions between endothelial cells, cancer cells, and other stromal cells to increase and improve tumor angiogenesis and thus tumor growth.

Altogether, these data show that E2 optimizes blood supply to ER-negative cancer cells through an important modification of tumor vasculature that seems normalized as described by Carmeliet and Jain (26, 37). This concept of normalization was documented by Mazzone and colleagues (26) who observed that tumor vessel normalization improves tumor perfusion and oxygenation and decreases metastasis. Importantly, hypoxia is a negative prognostic factor associated to chemoresistance and is described to promote tumor invasion and metastatic dissemination (37–40). As vessel normalization emerges actually as a potential therapeutic option (25, 37), the stromal ERα-mediated impact of E2 on ER-negative cancer cell growth and angiogenesis is of particular interest. Indeed, even if E2 indirectly contributes to promote ER-negative cancer cell growth, the decreased tumor hypoxia and necrosis conferred by E2 could present a therapeutic advantage in terms of tumor response to chemotherapy in hematopoietic lineage, in pericytes, fibroblasts/myofibroblasts (42–44). Tie2-expressing monocytes have been shown to promote tumor angiogenesis in various mouse tumor models (45). In addition, bone marrow–derived endothelial progenitor cells (EPC) could also have contributed to the formation of tumor endothelium, but it remains highly controversial (29). In this work, E2 elicited similar protumoral effects in chimeric mice irrespective of the presence or absence of ERα in bone marrow–derived cells. Moreover, the protumoral effect of E2 was abrogated in chimeric mice harboring ERα-negative Tie2-expressing cells, but with ERα-positive hematopoietic cells, showing a minor role of E2 on bone marrow–derived EPC in tumor vasculature development. This result corroborates studies showing that tumor endothelium does not predominantly originate from bone marrow cells, particularly in C57BL/6 mice (46, 47). As we showed that the protumoral effect of E2 relies on ERα-dependent promotion and improvement of angiogenesis, this is in line with the crucial role played by endothelial ERα in the various effects exerted by E2 on endothelium, that is, endothelial cell migration and proliferation in vitro, reendothelialization acceleration in vivo (5, 23, 48–50) and with the major contribution of pericytes to vessel stabilization (26, 37, 40). Thus, Tie2-positive cells expressing ERα, but not bone marrow ERα, are necessary to promote the E2-induced growth of ERα-negative tumors.

Finally, we clearly detected ERα in human stromal cells surrounding human ER-positive and also ER-negative breast cancers. These stromal ERα-positive cells were negative for CD45 and SMA and were rarely positive for CD31. However, the lack of selective fibroblast markers led the phenotype of the stromal ERα-positive cells difficult to be clearly identified. Until now, this stromal expression of ERα is not routinely evaluated during diagnosis of human breast tumors. Nevertheless, it could be relevant if a correlation between stromal ERα expression, prognosis, and/or treatment response could be addressed in women.

In summary, we show here that ERα of microenvironment plays a crucial role in the in vivo growth of ERα-negative cancer cells under E2 treatment. Indeed, stromal ERα is necessary to induce tumor growth, mediating adaptation of tumor angiogenesis and vessel stabilization that subsequently improves oxygen and nutrients delivery, thereby preventing hypoxia and necrosis. This could have implications in the management of patients, particularly in the diagnosis and the schedule of hormono-, radio-, and chemotherapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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